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Calpain inhibitor A-558693 in experimental focal cerebral ischemia in rats

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Objectives: *Calpains are intracellular proteases, which are activated in various cerebral injuries. We studied the expression of μ-calpain in a model of focal cerebral ischemia/reperfusion and the efficacy of the calpain inhibitor A-558693.*
Methods: *A transient occlusion of the middle cerebral artery was produced in male Wistar rats by using the suture model with 3 hours of ischemia and 24 hours of reperfusion. Six animals were given the calpain inhibitor and six animals were treated with placebo. The infarct size was determined by the loss of the calpain substrate microtubule-associated protein-2 (MAP-2) immunohistochemistry using volumetry in serial slices of the brains. Furthermore μ-calpain positive-stained cells were detected by immunohistochemistry and western blotting.*
Results: *In placebo-treated animals the μ-calpain expression was significantly increased in the ischemic hemisphere compared with the contralateral non-ischemic hemisphere (88.6 versus 10.5% in the basal ganglia, 60.7 versus 10.7% in the cortex, $p<0.001$, respectively) with a subsequent loss its substrate MAP-2. However, the use of the calpain inhibitor A-558693 did not significantly change the μ-calpain expression, nor significantly reduce the infarct volume.*
Discussion: *The present data indicate that μ-calpain proteolysis plays an important role in the chain of events following cerebral ischemia. However, the calpain inhibitor A-558693 failed to prevent these changes.* [Neurol Res 2005; **27**: 466–470]

Keywords: Calpain inhibitor; middle cerebral artery occlusion; immunohistochemistry; MAP-2

INTRODUCTION

Various mechanisms are involved in the pathophysiological alterations that result from focal cerebral ischemia. These include impaired cerebral blood flow, increased intracellular levels of Na^+ and Ca^{2+} and cytoskeletal degradation[1–4]. The increase of intracellular Ca^{2+} especially contributes to an activation of some members of the calpain family that consist of neutral cysteine proteases[5]. Calpains represent a family of non-lysosomal cysteine proteases of which several members have been reported. The most important calpains consist of μ-calpain and m-calpain. The proteolytic activity of μ-calpain, which needs micromolar concentrations of calcium for activation, and also m-calpain, which needs millimolar concentrations of calcium, substantially increases after ischemic[6,7] or traumatic[8] brain injury. Activated calpain affects several substrates, many of which are cytoskeletal proteins such as spectrin, microtubule-associated protein 2 (MAP-2), as well as the neurofilament proteins NF68 and NF200[9–12].

Global inhibition of these proteases might have beneficial effects in focal cerebral ischemia. This was recently shown by our group in a model of cerebral ischemia, in which hypothermia reduced the μ-calpain expression in the ischemic hemisphere[13]. In addition pharmacological inhibition of calpains has been of interest in drug discovery research for many years, however up to now, no clinical trial investigating the effect of calpain inhibitors has been reported. Nevertheless, in experimental studies calpain inhibition could lead to a significant reduction in infarct size after focal cerebral ischemia. For example, it has been shown that administration of the calpain inhibitor Cbz-Val-Phe-H before ischemia can significantly reduce the infarct volume[14]. Another calpain inhibitor (AK 295) was shown to be effective even several hours after initiation of cerebral ischemia[15–17]. However, most of the investigated calpain inhibitors are not specific against calpain, and therefore the observed effect might be due to a broad inhibition of several proteases.

The aim of our study was to show the immunohistochemical expression of μ-calpain and its effect on the substrate MAP-2 in an experimental model of focal

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cerebral ischemia in rats and to evaluate the efficacy of A-558693, a novel specific benzoylalanine-derived ketoamide calpain inhibitor[18], when administered 150 minutes after initiation of ischemia.

METHODS

Rat model of transient focal cerebral ischemia

Adult male Wistar rats were anesthetized with thiopental (100 mg/kg intraperitoneally), tracheotomized, and artificially ventilated with an animal ventilator. The right femoral vein was cannulated to provide fluid substitution. A catheter was inserted into the right femoral artery to allow the continuous measurement of arterial blood pressure and blood gases. The rats were kept at a constant temperature of ~37°C using a feedback heating pad.

Transient focal cerebral ischemia was induced by a modified method of intraluminal vascular occlusion[19]. Briefly, the left common carotid artery was ligated and cannulated with a polyethylene tube (Ethicon, Norderstedt, Germany), through which a 3-0 surgical nylon suture with a thickened tip was inserted ~28–30 mm from the bifurcation of the common carotid artery, toward the intracranial part of the internal carotid artery to occlude the middle cerebral artery (MCA). Reperfusion was initiated by retracting the suture toward the base of the skull and allowing recirculation via the circle of Willis. At the end of the experiment, the brain was perfused with a 1% albumin solution and was frozen at −80°C. The base of the skull was inspected to exclude hemorrhagic infarction. The brains were cut in a cryostat at −18°C, and 10-µm-thick sections were collected on pre-labeled, warm glass slides. After circa 50 consecutive sections, two sections were used for the volumetry, while the remaining sections were used for other examinations. All animal procedures were approved by the government of Upper Bavaria and were performed in accordance with the European Communities Council Directive. In accordance with these standards, every effort was made to reduce the number of animals used and to ensure that they were free of pain or discomfort.

Calpain inhibitor

The calpain inhibitor A-558693 was synthesized as described in detail[18]. The compound carries ketoamide as the functional group. The inhibition of human and rat µ-calpain is in the low nanomolar range with K_i values for the different species of 18.3 and 18.8 nM, respectively. The cysteine proteases cathepsin B and cathepsin L are inhibited between 5- and 8-fold less, while the yeast proteasome is not even inhibited at 100 µM concentrations by this compound. A-558693 shows considerable water-solubility at neutral pH-values, which enabled the intravenous application of aqueous solutions.

Experimental groups

Two experimental groups were set up. The first group of six animals served as controls, receiving no calpain inhibitor. The second group of six animals was treated with the calpain inhibitor A-558693. The inhibitor was given in a dose of 10 mg/kg body weight as a bolus over 5 minutes, 150 minutes after initiation of ischemia, and 30 minutes before starting reperfusion of the MCA. Afterwards a continuous infusion of 5 mg/kg body weight per hour was administered for 24 hours. All animals were subjected to 3 hours of ischemia followed by 24 hours of reperfusion.

Antibodies and immunohistochemistry

Neuronal injury was detected by immunohistochemical staining using a monoclonal antibody against MAP-2 (Boehringer Mannheim, Germany). MAP-2 is a cytoskeletal protein, which is degraded by activated proteinases in the early phases of ischemia. While the antibody used does not detect digested protein, areas with neuronal damage show a loss of staining. These areas of MAP-2 demarcation were defined as regions of interest, in which calpain activation was evaluated and compared with the contralateral hemispheres. Consecutive sections underwent immunohistochemical staining for µ-calpain to detect the presence of intracellular proteases in these defined areas. Calpain-positive cells were identified with a polyclonal rabbit µ-calpain antibody against the inactive large subunit (80 kDa, R&D Laboratories, Munich, Germany).

Sections were fixed with acetone/chloroform for 5 minutes at +4°C and immersed in 100 mM/l glycine in phosphate buffer solution (PBS) for 10 minutes. Sections were rinsed in PBS wash solution and then incubated with Blotto for 20 minutes to reduce non-specific binding. Each section was incubated with 150 µl of the primary antibody solution for over 2 hours at 37°C. The working concentration of the primary antibody was 1 : 50 for µ-calpain and 1 : 800 for MAP-2. After the sections had been washed with PBS, they were incubated with either biotinylated goat serum against rabbit IgG or biotinylated horse serum against mouse IgG for 30 minutes at 37°C (Vector Laboratories). Vectastain ABC reagent was added for 30 minutes at 37°C after rinsing the sections with PBS. Chromogen (AEC Kit Biomeda Corp.) was used to develop the peroxidase signal. All sections were counterstained with Mayer's Hematoxylin (Sigma) for 30 seconds, blued in saturated sodium bicarbonate, and mounted on crystal slides. Negative and positive controls were routinely performed in each staining experiment.

Videoimaging and microscopy

For the analyses light microscopy was used (Zeiss, Axioskop 2, Germany), together with a photo camera (Sony, PowerHAD, Japan). The numbers of peroxidase-stained cells were calculated with a computerized videoimaging system (Optimas Software, Version 6.5 from Media Cybernetics Silver Spring, USA). To determine the calpain-positive cells, the relative intensity of the staining coefficient algorithm (RISC) was used[20]. RISC was determined by calculating the decimal

logarithm of the quotient of the background staining to the staining by calpain-positive cells.

Western blotting

Protein isolation and western blotting were performed as described elsewhere[21]. Blots were first incubated with rabbit anti-μ-calpain antibodies at a dilution of 1:1000 (R&D Laboratories, Munich, Germany), and subsequently with biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA). Each blot is representative of at least three experiments. An optical analysis program (Tina version 2.08, Raytest Isotopenmessgeräte GmbH, Munich, Germany) was used to compare the different bands by optical densitometry. Results were displayed on an arbitrary optical density scale. To allow comparison over multiple samples run in different gels, the amount of proteins in the ischemic side was normalized by dividing it by the amount of protein in the non-ischemic side. Ischemic and non-ischemic material from the same animal was analyzed in the same gel to allow unbiased calculation of the ratio ischemic side to non-ischemic side.

Volumetry

To calculate the lesion volume, all sections for volumetry with MAP-2 immunohistochemistry were first scanned using a standard flatbet scanner. The files were imported into Optimas 6.5-imaging software, the lesioned areas delineated, and the size converted to the metric system. Next, for every detection method, the partial volume between two adjacent sections was computed using the formula for a conic section:

$$(\text{Area}_{i-1} + \text{Area}_i + \sqrt{\text{Area}_{i-1} \times \text{Area}_i})/3 \times (\text{distance between sections})_{i-1,i}$$

with $\text{Area}_{1,2,3,\ldots,n-1}$ being the area of the lesion of the serial sections detected by the same method, and Area_0 and Area_n are the first and last section without a lesion. Then all partial volumes were added to find the total lesion volume[22].

Statistical analysis

Data were expressed as mean ± SD. Comparisons between values from infarcted and control areas as well as comparisons of cerebral infarct size and calpain breakdown between the calpain inhibitor and placebo-treated animals were made with the *t*-test using a level of significance of 5%.

RESULTS

Expression of μ-calpain in cerebral ischemia by immunohistochemistry and western blotting

Calpain-positive-stained cells were rarely determined in the non-ischemic hemisphere. Basal ganglia and the cortex of placebo-treated animals showed a significant increase of calpain expression on the ischemic side (88.6 versus 10.5% in basal ganglia, 60.7 versus 10.7% in the cortex, $p<0.001$, respectively). The western blot analysis for anti-μ-calpain revealed a significant increase of calpains in the ischemic compared with the non-ischemic hemisphere. The increase of calpain in the cortex as detected by western blot was 141 ± 11%, and in the ischemic basal ganglia 164 ± 26%.

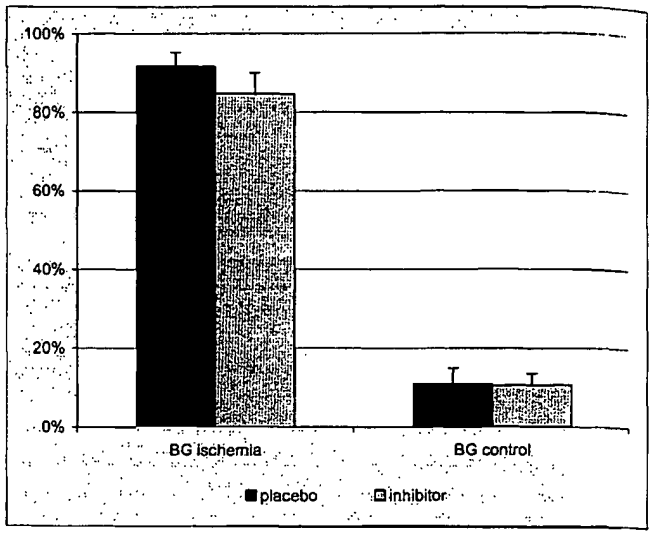

Figure 1: Ratio of calpain-positive cells to the total number of cells in the basal ganglia after 3 hours of ischemia and 24 hours of reperfusion. The left columns indicate the ischemic side, and the right columns the non-ischemic side. The black columns indicate the placebo-treated animals, whereas the gray columns represent the calpain-inhibitor-treated group. The difference was not significant in the ischemic or non-ischemic side

Effect of calpain inhibitor A-558693 on the calpain expression and the infarct size

A-558693-treated animals showed a trend for fewer calpain-positive cells in the basal ganglia of the ischemic side. RISC in the basal ganglia was 0.22 ± 0.09 in animals receiving the calpain inhibitor (CI) and 0.34 ± 0.10 in the placebo-treated animals ($p=0.06$). The RISC in the cortex of CI rats was 0.11 ± 0.07 compared with 0.22 ± 0.09 in placebo-treated animals (n.s.). The ratio of calpain-positive and total cells in the basal ganglia and the cortex did not significantly differ between these two groups (*Figures 1* and *2*).

The western blot analysis for anti-μ-calpain revealed no significant difference between the A-558693-treated animals in the ischemic hemisphere compared with the placebo-treated animals, whether in the cortex or in the basal ganglia. The amount of calpain in the cortex was increased to 170 ± 32% in A-558693, and to 141 ± 11% in the placebo-treated animals (n.s.). The increase of calpain in the ischemic basal ganglia of the calpain-inhibitor group was 171 ± 30% compared with 164 ± 26% in the placebo group (n.s.), see *Figure 3*.

To determine the infarct volume, MAP-2 a substrate of the calpains was used. MAP-2 immunohistochemistry revealed that the infarct volume, determined by volumetric analysis based on a conic section, in the A-558693-treated animals tended to be smaller than in the control animals (148 ± 84 versus 207 ± 110 mm^3). Due

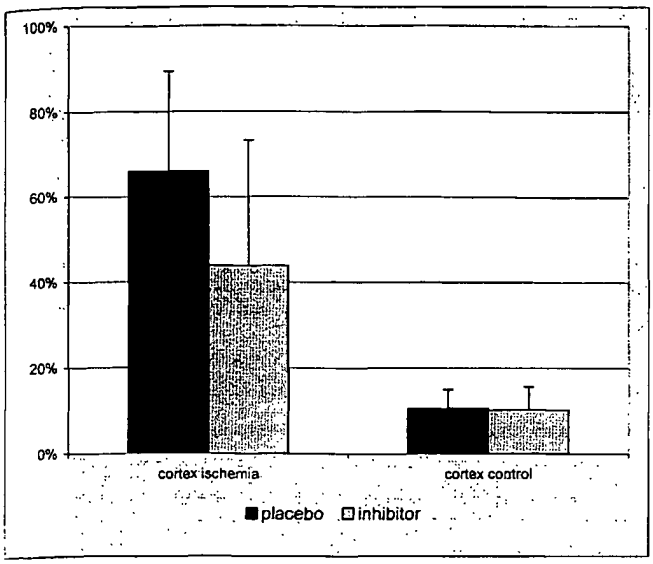

Figure 2: Ratio of calpain-positive cells to the total number of cells in the cortex after 3 hours of ischemia and 24 hours of reperfusion. The left columns indicate the ischemic side, and the right columns the non-ischemic side. The black columns indicate the placebo-treated animals, whereas the gray columns represent the calpain inhibitor-treated group. The difference was not significant in the ischemic or non-ischemic side

to a large standard deviation this difference was not significant (p=0.34).

DISCUSSION

The main finding of this study is that the calpain inhibitor A-558693, administered 150 minutes after initiation of ischemia, had no significant effect on the infarct size or the expression of calpain in this animal model of focal cerebral ischemia. However, there was a trend to smaller infarcts and also fewer calpain-positive cells in the calpain-inhibitor group.

Our data on the increased calpain expression in the ischemic area agree with the breakdown of the calpain substrate MAP-2 in the placebo group, as well as in the calpain-inhibitor-treated group. The calpain-positive cells were mainly detected in the basal ganglia, but also in the cortex on the ischemic side. This pattern of μ-calpain expression is in accordance with the time-dependent increase of m-calpain[7] and μ-calpain[13] expression in focal cerebral ischemia in rats and the increase of μ-calpain in an animal model of cerebral venous thrombosis[23]. The increase in μ-calpain expression in the ischemic area was accompanied by a loss of its substrate MAP-2. It is well known that prolonged calpain-mediated breakdown of substrates occurred after experimental traumatic brain injury or after focal cerebral ischemia in rats[6,24], thus our data are in accordance with these reports.

The calpain inhibitor A-558693 had no significant effect on the infarct size or on the expression of calpains after 150 minutes of ischemia and 24 hours of reperfusion, despite a trend to smaller infarcts and less calpain-positive cells. Our results contradict those of other studies, reporting a reduction of infarct size by calpain

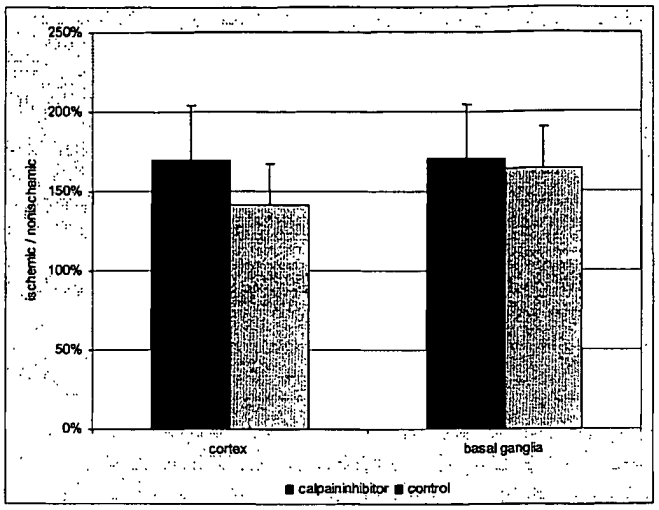

Figure 3: Calpain expression measured by western blot. The black columns indicate calpain-inhibitor and the gray boxes indicate placebo-treated animals. The left side represents the calpain expression in the cortex and the right side the calpain expression in the basal ganglia. Differences were not significant for cortex or basal ganglia

inhibitors. When MDL28170/Cbz-Val-Phe-H was given 30 minutes before vessel occlusion, the rats exhibited significantly smaller volumes of cerebral infarction[14]. Bartus and co-workers also showed that the administration of calpain inhibitors AK 275 and AK 295 after initiation of ischemia can reduce the infarct volume up to 75 and 30%, respectively[15,16]. Markgraf and his group postulated a period of ~6 hours for calpain inhibition in focal cerebral ischemia in rats[17]. All of these studies measured the infarct volume by the TTC method, a macroscopic method that does not permit evaluation of cell damage on a microscopic scale. In contrast, our results were based on a more detailed histological analysis of the post-ischemic damage. We determined the volumetric infarct by the breakdown of the calpain substrate MAP-2, and also performed a quantitative analysis of the calpain expression in the ischemic hemisphere by immunohistochemistry and western blotting. Despite these various techniques for identifying cerebral injury we were not able to show any significant effect of the calpain inhibitor. In contrast to our data, one group showed a reduction of the necrotic cells in the cortex in an animal model of global cerebral ischemia using the calpain inhibitor MDL28170, although these authors did not directly assess the effect on the calpain expression[25].

There might be several reasons for our failure to demonstrate the efficacy of this new calpain inhibitor A-558693. First, the calpain inhibitor A-558693 is a very potent and specific inhibitor against calpains. This is in contrast to other inhibitors with a broader spectrum and inhibition of other proteases, like cathepsins. Therefore, inhibition of calpains might not be effective enough to stop the progression of the infarct size. However, specific calpain inhibition might be an effective strategy in other cerebral diseases like traumatic brain injury, as one study was able to demonstrate a reduction in the

total numbers of damaged neurons by 41% by the use of a ketoamid-calpain inhibitor[18]. Another reason might be that the calpain inhibitor was given 150 minutes after ischemia, while most of the previous studies administered the inhibitor before or shortly after initiation of ischemia. However, one study showed a significant reduction of infarct size even 6 hours after initiation of ischemia with the calpain inhibitor MDL28, 170. But as mentioned before MDL28, 170 has a more broad inhibitory effect and might be therefore effective even after late administration.

Our study has some limitations. First, the administration of the calpain inhibitor 150 minutes after initiation of ischemia seems to be too late, as the time window for this specific calpain inhibitor might be shorter; however, we tried to be as close to human settings, and therefore administration before or shortly after initiation of ischemia might be effective, but clinically irrelevant. Secondly, we cannot exclude that the determination of the infarct size by one substrate of the calpains will be representative for the infarct size. Therefore, our tissue-saving volumetric access of determination of the infarct size might not be strictly comparable with other studies that showed a reduction in infarct volume, for example by TTC staining. However, in our previous study we found a good correlation between the loss of MAP-2 and infarct size using MRI[22]. In addition, as MAP-2 is a substrate of activated calpains, the effect of calpain inhibitors might be even more pronounced by determination of directly related substrates. Therefore, we consider the loss of MAP-2 to be a relevant post-ischemic event.

The present data indicate that μ-calpain proteolysis plays an important role in the chain of events following experimental cerebral ischemia and the subsequent loss of its substrate MAP-2. On the basis of measurements of infarct volumetry by the loss of the calpain substrate MAP-2, the expression of calpains by immunohistochemistry and western blotting, we were not able to establish a neuroprotective effect of the calpain inhibitor A-558693 in our model.

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REFERENCES

1 Croall DE, DeMartino GN. Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. *Physiol Rev* 1991; 71: 813–847
2 Takagaki Y, Itoh Y, Aoki Y, *et al.* Inhibition of ischemia-induced fodrin breakdown by a novel phenylpyrimidine derivative NS-7: An implication for its neuroprotective action in rats with middle cerebral artery occlusion. *J Neurochem* 1997; 68: 2507–2513
3 Saatman KE, Bozyczko CD, Marcy V, *et al.* Prolonged calpain-mediated spectrin breakdown occurs regionally following experimental brain injury in the rat. *J Neuropathol Exp Neurol* 1996; 55: 850–860
4 Siman R, Card JP, Davis LG. Proteolytic processing of beta-amyl precursor by calpain I. *J Neurosci* 1990; 10: 2400–2411
5 Zivin JA, Choi DW. Stroke therapy. *Sci Am* 1991; 265: 56–63
6 Bartus RT, Dean RL, Cavanaugh K, *et al.* Time-related neuro changes following middle cerebral artery occlusion: Implicatic for therapeutic intervention and the role of calpain. *J Cereb Blo Flow Metab* 1995; 15: 969–979
7 Liebetrau M, Staufer B, Auerswald EA, *et al.* Increased intracellu calpain detection in experimental focal cerebral ischem *Neuroreport* 1999; 10: 529–534
8 Kampfl A, Posmantur RM, Zhao X, *et al.* Mechanisms of calp proteolysis following traumatic brain injury: Implications pathology and therapy: A review and update. *J Neurotrau* 1997; 14: 121–134
9 Saido TC, Sorimachi H, Suzuki K. Calpain: New perspectives molecular diversity and physiological-pathological involveme *FASEB J* 1994; 8: 814–822
10 Dawson DA, Hallenbeck JM. Acute focal ischemia-induc alterations in MAP2 immunostaining: Description of tempc changes and utilization as a marker for volumetric assessment acute brain injury. *J Cereb Blood Flow Metab* 1996; 16: 170–1
11 Posmantur R, Kampfl A, Siman R, *et al.* A calpain inhibi attenuates cortical cytoskeletal protein loss after experimer traumatic brain injury in the rat. *Neuroscience* 1997; 77: 875–8
12 Lofvenberg L, Backman L. Calpain-induced proteolysis of be spectrins. *FEBS Lett* 1999; 443: 89–92
13 Liebetrau M, Burggraf D, Martens HK, *et al.* Delayed moder hypothermia reduces calpain activity and breakdown of substrate in experimental focal cerebral ischemia in r *Neurosci Lett* 2004; 357: 17–20
14 Hong SC, Goto Y, Lanzino G, *et al.* Neuroprotection with a calp inhibitor in a model of focal cerebral ischemia. *Stroke* 1994; 663–669
15 Bartus RT, Baker KL, Heiser AD, *et al.* Postischemic administrat of AK275, a calpain inhibitor, provides substantial protect against focal ischemic brain damage. *J Cereb Blood Flow Me* 1994; 14: 537–544
16 Bartus RT, Hayward NJ, Elliott PJ, *et al.* Calpain inhibitor AK protects neurons from focal brain ischemia. Effects of postoc sion intra-arterial administration. *Stroke* 1994; 25: 2265–2270
17 Markgraf CG, Velayo NL, Johnson MP, *et al.* Six-hour window opportunity for calpain inhibition in focal cerebral ischemia rats. *Stroke* 1998; 29: 152–158
18 Lubisch W, Beckenbach E, Bopp S, *et al.* Benzoylalanine-deri ketoamides carrying vinylbenzyl amino residues: Discovery potent water-soluble calpain inhibitors with oral bioavailabil *J Med Chem* 2003; 46: 2404–2412
19 Longa EZ, Weinstein PR, Carlson S, *et al.* Reversible mid cerebral artery occlusion without craniectomy in rats. *Stroke* 19 20: 84 91
20 Moller JC, Klein MA, Haas S, *et al.* Regulation of thrombospon in the regenerating mouse facial motor nucleus. *Glia* 1996; 121–132
21 Hamann GF, Liebetrau M, Martens H, *et al.* Microvascular b lamina injury after experimental focal cerebral ischemia reperfusion in the rat. *J Cereb Blood Flow Metab* 2002; 22: 5 533
22 Kloss CU, Thomassen N, Fesl G, *et al.* Tissue-saving inf volumetry using histochemistry validated by MRI in rat fc ischemia. *Neurol Res* 2002; 24: 713–718
23 Liebetrau M, Gabricjcic-Geiger D, Meyer P, *et al.* Increa calpain expression following experimental cerebral venous thr bosis in rats. *Thromb Res* 2003; 112: 239–243
24 Pettigrew LC, Holtz ML, Craddock SD, *et al.* Microtub proteolysis in focal cerebral ischemia. *J Cereb Blood Flow Me* 1996; 16: 1189–1202
25 Li PA, Howlett W, He QP, *et al.* Postischemic treatment v calpain inhibitor MDL 28170 ameliorates brain damage in a ge model of global ischemia. *Neurosci Lett* 1998; 247: 17–20

Limited Access Trial Using Amifostine for Protection Against Cisplatin- and Three-Hour Paclitaxel–Induced Neurotoxicity: A Phase II Study of the Gynecologic Oncology Group

By David H. Moore, James Donnelly, William P. McGuire, Lois Almadrones, David F. Cella, Thomas J. Herzog, and Steven E. Waggoner

Purpose: **The purpose of this study was to determine whether amifostine (WR-2721) prevents or ameliorates clinically significant (grade 2 to 4) neurotoxicity associated with cisplatin and 3-hour paclitaxel chemotherapy.**

Materials and Methods: **The chemotherapy program consisted of intravenous paclitaxel 175 mg/m^2 over 3 hours followed by amifostine 740 mg/m^2 and cisplatin 75 mg/m^2 administered over 90 minutes beginning 15 minutes after amifostine administration. At baseline, before each treatment cycle, and for 3 months after completing chemotherapy, patients were evaluated for evidence of neurotoxicity and other treatment-related adverse effects using three methods: standard clinical evaluation (National Cancer Institute common toxicity criteria [CTC] grading), a neurotoxicity questionnaire to assess symptoms and limitations imposed by peripheral neuropathy, and vibration perception threshold (VPT) testing.**

Results: **Four of 27 assessable patients developed grade 2 to 4 neurotoxicity based on clinical assessments and CTC grading. This number of neuropathic events exceeded the predetermined threshold level for a second stage of accrual and the study was closed.**

Conclusion: **Amifostine's level of activity in this trial was insufficient to warrant further study in a phase III trial. Based on the receiver operating characteristic analysis, it would appear that VPT measurements are less sensitive to the development of peripheral neuropathy than the neurotoxicity questionnaire. The questionnaire, referred to as the Functional Assessment of Cancer Therapy/Gynecologic Oncology Group–Neurotoxicity, may be used instead of VPT measurements in future studies of chemotherapy-induced peripheral neuropathy.**

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SINCE ITS introduction two decades ago, platinum-based chemotherapy has become the standard treatment for epithelial ovarian cancer.[1] Prospective controlled trials have confirmed the superiority of cisplatin-containing regimens over drug combinations that do not contain cisplatin.[2-4] Paclitaxel was identified as a drug with significant activity against epithelial ovarian cancer approximately 15 years ago.[5] Scarce supply initially hampered its clinical development, but after this supply problem was alleviated, the National Cancer Institute (NCI) initiated a trial of paclitaxel as salvage treatment for patients with platinum-refractory ovarian cancer. Among more than 1,000 patients who participated in this important study, objective responses were reported in 22% of patients (4% complete response; 18% partial response).[6]

Cisplatin and paclitaxel were successfully combined in a phase I trial in which paclitaxel was given as a 24-hour infusion, preferably before cisplatin administration.[7] Subsequently, the Gynecologic Oncology Group (GOG) completed a phase III trial of cisplatin plus paclitaxel versus cisplatin plus cyclophosphamide in patients with suboptimal stage III/IV ovarian cancer. The paclitaxel-containing regimen yielded superior objective response rates (73% *v* 60%), progression-free survival (median, 18 *v* 13 months), and overall survival (median, 38 *v* 24 months).[8] Paclitaxel has subsequently been combined with both cisplatin and carboplatin at various dose schedules and infusion rates. Pending results from ongoing and planned phase III investigations, a paclitaxel plus platinum combination is considered the preferred treatment for epithelial ovarian cancer.

One of the dose-limiting side effects of cisplatin chemotherapy is peripheral neuropathy manifested by paresthesias, loss of deep tendon reflexes, and decreased sensory capabilities (fine touch, vibration perception, proprioception). Lo Monaco et al[9] conducted prospective neurophysiologic investigations in patients undergoing cisplatin chemotherapy. Study patients underwent serial electromyography assessments of distal limb muscles and motor and sensory nerve conduction studies. The incidence of polyneuropathy was 44% at a cumulative cisplatin dose of 400

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mg/m^2 and 88% at a cumulative dose of 600 mg/m^2. At 3 months posttreatment, all patients had evidence of peripheral neuropathy. One patient experienced grade 1 and the remainder had grade 2 to 4 neurotoxicity.[9] Paclitaxel may also induce a peripheral neuropathy characterized by numbness and paresthesias in a stocking-glove distribution.[10] In a phase I study of paclitaxel with granulocyte colony-stimulating factor support, peripheral neurotoxicity was the dose-limiting toxicity.[11] At conventional doses, the neurotoxicity of paclitaxel is believed to be rare. The GOG reported that peripheral neuropathy was more common in the cisplatin plus paclitaxel (*v* cyclophosphamide) arm but was generally mild.[8] When cisplatin was combined with paclitaxel (135 or 175 mg/m^2) administered as a 3-hour infusion, Markman et al[12] noted a high incidence of neurotoxicity, with 16 (42%) of 38 patients experiencing grade 2 or worse peripheral neuropathy. This was similar to the findings of the NCI of Canada Clinical Trials Group, which reported a 49% incidence of neurosensory toxicity with 3-hour paclitaxel in a cohort of patients with recurrent ovarian cancer after one or two prior platinum-containing regimens.[13] Piccart et al[14] reported the results of a randomized trial of cisplatin plus paclitaxel versus cisplatin plus cyclophosphamide that was conducted as an intergroup collaboration between European and Canadian investigators. Paclitaxel was given at a dose of 175 mg/m^2 over 3 hours and followed by cisplatin 75 mg/m^2. During the first six treatment cycles, 14% of patients who received the paclitaxel-containing regimen experienced grade 3 to 4 neurosensory toxicity.

Amifostine (WR-2721) is an organic thiophosphate that was originally developed as a radioprotective compound. It is dephosphorylated in tissues to an active free thiol metabolite that has been shown to reduce the toxic effects of cisplatin.[15] Animal and clinical studies have suggested that amifostine can reduce the neurotoxicity of cisplatin chemotherapy.[16,17] In a randomized, controlled trial of cisplatin plus cyclophosphamide with or without amifostine, Rose et al[18] reported a significant reduction in peripheral neuropathy and a 43% reduction in ototoxicity in the amifostine-treated group. Side effects associated with amifostine administration include transient hypotension during drug infusion, nausea and emesis, hypocalcemia, and (rarely) allergic reactions. Patients should be adequately hydrated before amifostine infusion, and frequent blood pressure monitoring is advised.

Spurred by the current push toward outpatient treatment, the 3-hour infusion of paclitaxel has become increasingly popular. It is the purpose of this study to determine whether amifostine can reduce the potential for clinically significant (grade 2 to 4) neurotoxicity associated with cisplatin and 3-hour paclitaxel chemotherapy to 5% from an anticipated frequency of 15%.

MATERIALS AND METHODS

Patients

This phase II study (Protocol 9805) was conducted by selected GOG institutions and their affiliates. Eligible patients included women with epithelial ovarian, primary peritoneal, fallopian tube, endometrial, or cervical carcinoma or uterine sarcoma for whom the proposed treatment was cisplatin plus paclitaxel chemotherapy. Potential study participants were counseled regarding the possible adverse effects of the proposed protocol therapy, as well as the availability of alternative treatments, including carboplatin-containing regimens. Patients could not be eligible for a higher priority GOG study and were ineligible if they had previously received radiation therapy or chemotherapy. All patients had a GOG performance status of 0 to 2, adequate bone marrow function (WBC $\geq$ 3,000/μL, granulocyte count $\geq$ 1,500/μL, platelet count $\geq$ 100,000/μL), renal function (serum creatinine $\leq$ 2.0 mg/dL), and hepatic function (bilirubin $\leq$ 1.5 times institutional normal, AST and alkaline phosphatase $\leq$ three times institutional normal), with no history of neuropathy, and without evidence of significant infection. Patients with a prior history of malignancy were eligible if they had received no radiation therapy or chemotherapy and were without evidence of recurrent cancer for a minimum of 12 months subsequent to diagnosis. The protocol was reviewed and approved by respective institutional review boards (or equivalent), and all study participants provided signed informed consent satisfying national, state, and local guidelines before the initiation of protocol treatment.

Treatment

The chemotherapy program consisted of intravenous paclitaxel 175 mg/m^2 administered over 3 hours followed by amifostine 740 mg/m^2 over 10 minutes, and cisplatin 75 mg/m^2 was administered over approximately 90 minutes beginning 15 minutes after administration of amifostine. Because of the propensity for amifostine to cause transient hypotension, patients were asked to temporarily discontinue antihypertensive medications for a minimum of 24 hours before each treatment course. Each treatment was preceded by a prophylactic regimen of corticosteroids, diphenhydramine, cimetidine, and either ondansetron or granisetron. Vigorous intravenous hydration was initiated at least 4 hours before chemotherapy drug administration, and during the amifostine infusion patients were kept in a recumbent position with blood pressure monitored every 5 minutes. Treatment modifications for patients who experienced adverse effects were specified in the protocol. Pending response to chemotherapy and resolution of any significant toxicities, treatments were administered every 3 weeks for a total of six cycles.

At baseline, before each treatment cycle, and 3 months after completing treatment, patients were evaluated by three methods for evidence of neurotoxicity and other treatment-related adverse effects. A clinical evaluation for neuropathic sensory toxicity was conducted and graded, using the NCI common toxicity criteria (CTC) scale. Patient-reported neurotoxicity symptoms were further assessed using the neurotoxicity subscale of the Functional Assessment of Cancer Therapy/Gynecologic Oncology Group–Neurotoxicity (FACT/GOG-Ntx), version 4.[19] The FACT/GOG-Ntx is a 39-item self-report questionnaire that contains two components: a general measure of quality of life (FACT-G) plus a neurotoxicity (Ntx) subscale.[20,21] Only the neurotoxicity subscale was used in the present study. The Ntx subscale is a validated, 11-item questionnaire designed to assess neuropathic side effects of platinum- and taxane-containing systemic chemotherapy experienced during the prior week (Fig 1). Scoring the measure includes reversing the item responses so that lower scores reflect more neurotoxicity; therefore, higher scores reflect better quality of life, consistent with all measures in the FACT system. This measure was chosen for its applicability to the cancer patient population, sound psychometric properties, ease of administration, and availability in many languages. Finally, patients were evaluated for evidence of peripheral neuropathy using the Vibratron II device (Physitemps Instruments Inc, Clifton, NJ) consisting of two vibrating rods located in separate units with cables connecting them to a controller unit with power supply, controller switches, and digital meter. The tandem vibrating rods are identical in appearance, and vibration is achieved by driving the transducers with a variable voltage source. A dual-position switch connected in series with the vibrating units controls which rod vibrates, while a dummy switch is used to imitate the sounds and motions of twitching. The amplitude of vibration is proportional to the square of the applied voltage and is continuously available on a digital display accurate to the nearest 0.1 units. The methodology of testing is a two alternative forced choice procedure, whereby at each trial, the patient is required to determine which of the two rods is actually vibrating. Under standardized testing conditions, a testing algorithm was followed to quantify the vibration perception threshold from the (same) index finger and great toe at the aforementioned time points.

GYNECOLOGIC ONCOLOGY GROUP **Appendix VI** **FACT/GOG-Ntx Subscale**

(1.)NAME (LAST) FIRST	(2.)GOG# (INST.,PROT.,SEQ.)	SECTION	(3.)DATE OF FORM
	9 8 0 5		

Instructions:

This is a list of statements that other people with your illness have said are important. By circling one number per line, please indicate how true each statement has been for you during the past 7 days. You will be asked to complete this form again, each time a course of chemotherapy is scheduled or given.

NTX Subscale of FACT/GOG-NTX (Version 4)

	ADDITIONAL CONCERNS	Not at all	A little bit	Some-what	Quite a bit	Very much
NTX 1	I have numbness or tingling in my hands.....	0	1	2	3	4
NTX 2	I have numbness or tingling in my feet.....	0	1	2	3	4
NTX 3	I feel discomfort in my hands.....	0	1	2	3	4
NTX 4	I feel discomfort in my feet.....	0	1	2	3	4
NTX 5	I have joint pain or muscle cramps.....	0	1	2	3	4
HI 12	I feel weak all over.....	0	1	2	3	4
NTX 6	I have trouble hearing.....	0	1	2	3	4
NTX 7	I get ringing or buzzing in my ears.....	0	1	2	3	4
NTX 8	I have trouble buttoning buttons.....	0	1	2	3	4
NTX 9	I have trouble feeling the shape of small objects when they are in my hand.....	0	1	2	3	4
An6	I have trouble walking.....	0	1	2	3	4

Fig 1. Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity (FACT/GOG-Ntx) subscale questionnaire.

Statistical Considerations

The primary goal of this study was to determine the proportion of patients treated with amifostine plus cisplatin and 3-hour paclitaxel chemotherapy who experience significant treatment-induced peripheral neuropathy. Grade 4 neurotoxicity constituting permanent sensory loss was not anticipated, given eligibility criteria, specified monitoring, and treatment modifications during therapy. For evaluation purposes, either of the following occurrences at any assessment point during the six-cycle treatment program was considered a significant event: grade 3 neuropathic-sensory toxicity or persistent grade 2 neuropathic-sensory toxicity requiring a dose reduction. If the event rate was $\geq$ 15%, then the proportion of patients treated with amifostine who experienced significant peripheral neuropathy while on the study regimen was considered too large to warrant further investigation. Alternatively, an event rate $\leq$ 5% would indicate that the proportion of patients treated with amifostine who experienced significant peripheral neuropathy is small enough to warrant further investigation in a phase III trial.

The anticipated annual accrual to this study was 20 patients. The study used a two-stage group sequential design. Twenty-nine patients were to be entered onto the first stage of the trial. If there were fewer than four events within the first 29 patients treated, then an additional 30 patients would be entered. At the conclusion of the second stage, if there were fewer than six events, then further investigation of amifostine in a phase III trial would be warranted. If the true event rate for this regimen is 5%, this design provides a 90% chance of concluding the regimen warrants further investigation at the end of the trial. If the true event rate for this regimen is 15%, then this design provides a 91% chance of concluding the regimen does not warrant further investigation at the end of the trial and a 65% chance of reaching this conclusion at the end of the first stage of accrual.

RESULTS

Five institutions participated in this limited access GOG study. The study opened in December 1998, and the first stage of accrual ended May 2000. Twenty-nine patients were enrolled, and all were subsequently confirmed eligible by central data and pathology committee review. Two patients prematurely discontinued chemotherapy because of medical complications, thus 27 patients were assessable for response and toxicity. The mean age of the study population was 61 years (range, 32 to 83 years). There were 18 patients with epithelial ovarian cancer, four patients with primary peritoneal carcinoma, two patients each with endometrial or fallopian tube carcinoma, and one patient with cervical carcinoma. Performance status in 10 patients was 0, in 16 patients was 1, and in one patient was 2.

Twenty-one patients completed all six cycles of cisplatin plus 3-hour paclitaxel chemotherapy and 3 months of clinical follow-

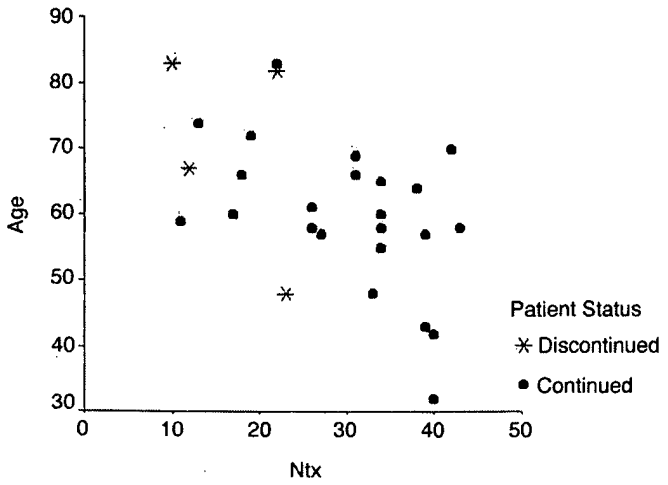

Fig 2. Patient age versus Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity subscale scores.

Table 1. Group Means for Ntx and VPT (by patient status in trial)

Status	No. of Patients	Mean	Standard Deviation	SE Mean
Finger VPT in μm				
Continue*	23	4.10	4.78	.10
Discontinue†	4	11.60	18.75	9.37
Toe VPT in μm				
Continue*	23	40.46	33.73	7.03
Discontinue†	4	48.15	54.24	27.12
Ntx				
Continue*	23	30.04	9.54	1.99
Discontinue†	4	16.75	6.70	3.35

NOTE. Nonparametric (Kolmogorov-Smirnov) exact test results: Ntx $P = .02$ (two-tailed); both finger and toe VPT not significant.

Abbreviations: Ntx, neurotoxicity; VPT, vibration perception threshold.

*Grade 0 to 1 neurotoxicity.

†Persistent grade 2 or grade 3/4 neurotoxicity.

up. Four patients completed the prescribed six cycles of chemotherapy but, at the time of analysis, 3 months of follow-up were not yet available. One patient had completed five cycles of chemotherapy and another had received two cycles of chemotherapy at the time the trial was suspended.

Four patients developed grade 2 to 4 neurotoxicity based on clinical assessments and CTC grading. Three patients experienced grade 3 peripheral neuropathy, and one patient experienced persistent grade 2 peripheral neuropathy despite reductions in chemotherapy dose. Because the number of neuropathic events exceeded the predetermined threshold for a second stage of accrual, the study was closed. The risk for chemotherapy-induced peripheral neuropathy was significantly greater among older patients (Fig 2). The correlation between patient age and peripheral neuropathy was identified with finger vibration perception threshold (VPT) measurements ($r = -0.40$; $P = .04$), toe VPT measurements ($r = -0.38$; $P = .05$), and FACT/GOG-Ntx subscale scores ($r = -0.53$; $P = .004$). The ages of the four patients who did not complete the trial were 48, 67, 82, and 83 years.

The neurotoxicity analysis was formulated from data obtained at the time of the last patient assessment, using all three tools: CTC grading, FACT/GOG-Ntx questionnaire, and Vibratron II VPT measurements. A comparison between index finger VPT, great toe VPT, and Ntx scores for patients who did (n = 4) versus those who did not (n = 23) experience clinically significant neurotoxicity is displayed in Table 1. There was no significant difference between mean VPT scores from either the index finger or great toe among patients who did versus did not experience ≥ grade 2 peripheral neuropathy. However, the difference in mean Ntx scores for patients who did versus those who did not experience ≥ grade 2 peripheral neuropathy was significant ($P = .02$). All four patients with dose-limiting neurotoxicity had Ntx scores less than 25.

To determine the predictive utility of VPT measurements and Ntx scores, a receiver operating characteristic (ROC) analysis was performed, and curves for all three measures are displayed in Fig 3. The analysis was conducted with the SPSS statistical package (Release 11.0.1; SPSS Inc, Chicago, IL). The diagonal line represents the null hypothesis that the signal detection of the instrument is equal to chance. The area under the curve for finger VPT, toe VPT, and FACT/GOG-Ntx measures is 0.57, 0.49, and 0.86, respectively, with only the FACT/GOG-Ntx area under the curve reaching statistical significance ($P = .02$). Given these data, it would seem that VPT measurements are less sensitive to the development of peripheral neuropathy than the FACT/GOG-Ntx subscale. The ROC analysis suggests that the Ntx subscale is an accurate indicator of the development of peripheral neuropathy, with sensitivity and specificity superior to an objective measure. Pending replication, this finding may be particularly notable because the criterion in the study was not a chronic, hardened neuropathy, but an emerging condition.

In Fig 4, the 95% confidence intervals for the FACT/GOG-Ntx means of patients who continued and discontinued the trial are shown. If clinical significance as defined by our CTC criterion is used as a gold standard, then a cutoff of 25 on the FACT/GOG-Ntx seems to be reasonably effective in discriminating significant neurotoxicity. In the present data, a score of 25 on the FACT/GOG-Ntx produces a sensitivity of 1 and a specificity of 0.85 with regard to trial completion. The correlation between the full CTC scale (grades 0 to 4) and the FACT/GOG-Ntx was as follows: Spearman's rho = -0.59 ($P = .001$), and examination of the plot of these scores showed a clear linear relationship.

DISCUSSION

Hay[22] conducted a MEDLINE search and found that only two studies (of 884 relevant study references) systematically evaluated the utility loss associated with severe chemotherapy-induced neuropathy. He further scrutinized data from 1,073 patients who participated in the Scottish Randomised Trial in Ovarian Cancer clinical trial of docetaxel plus carboplatin versus paclitaxel plus carboplatin. Severe neuropathy was associated with a 15% to 20% reduction in patient quality of life. The use of less neurotoxic regimens of otherwise equivalent effectiveness could therefore lead to improvements in patient-assessed health status and quality of life.[23]

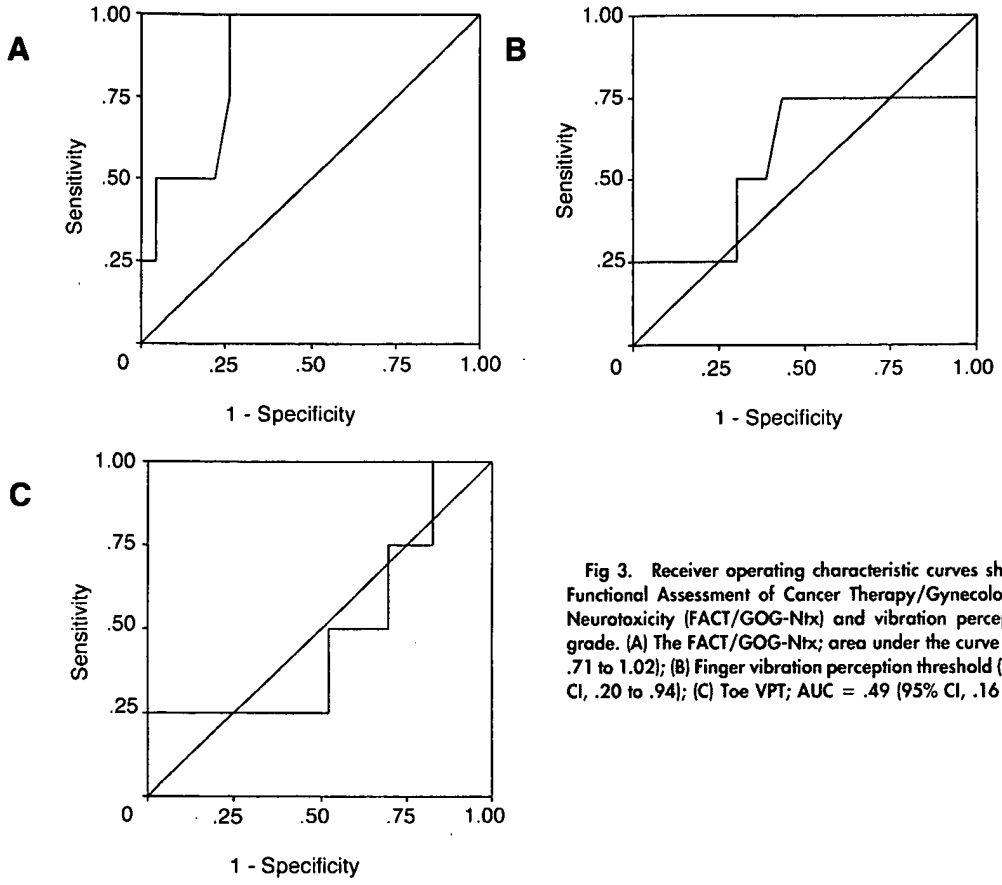

Fig 3. Receiver operating characteristic curves showing relationship of Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity (FACT/GOG-Ntx) and vibration perception to neurotoxicity grade. (A) The FACT/GOG-Ntx; area under the curve (AUC) = .86 (95% CI, .71 to 1.02); (B) Finger vibration perception threshold (VPT); AUC = .57 (95% CI, .20 to .94); (C) Toe VPT; AUC = .49 (95% CI, .16 to .82).

Several clinical trials have suggested that amifostine (WR-2721) may protect against cisplatin-induced peripheral neuropathy. Glover et al[24] reported results from a phase I trial of cisplatin plus WR-2721 in which cisplatin doses were increased from 50 mg/m^2 to 150 mg/m^2, and escalating doses of WR-2721 were administered 15 minutes before the cisplatin infusion. Grade 1 to 2 neuropathies developed in 26% of patients after a median cumulative cisplatin dose of 725 mg/m^2. These investigators subsequently conducted another phase I trial of escalating doses of cisplatin (60 to 150 mg/m^2) plus a fixed dose of amifostine (740 mg/m^2). Among 52 assessable patients, there were seven patients (13%) who developed mild to moderate peripheral neuropathy after a median cumulative cisplatin dose of 870 mg/m^2.[25] Mollman et al[17] reported results from a prospective study of cisplatin, alone or in combination with other chemotherapeutic agents. The overall incidence of neuropathy was 49%; however, among patients who also received WR-2721, the incidence of neurotoxicity was 25%. Furthermore, the mean dose at the onset of neuropathy was 635 mg/m^2 for patients who did, versus 383 mg/m^2 for patients who did not, receive WR-2721. In a prospective study of high-dose cisplatin plus WR-2721 for treatment of metastatic malignant melanoma, Glover et al[26] reported that nine (25%) of 36 assessable patients developed peripheral neuropathy at a median cumulative cisplatin dose of 670 mg/m^2.

Kemp et al[27] reported the results from a phase III trial of cisplatin 100 mg/m^2 plus cyclophosphamide 1,000 mg/m^2 with or without amifostine for treatment of epithelial ovarian cancer, in which 242 patients received six treatment cycles. There was no significant difference between the two arms in the incidence of ototoxicity. The overall incidence of grade 3 neuropathy was low (only 24 patients); nonetheless, there was a significantly lower incidence of peripheral neuropathy among patients who did versus those who did not receive amifostine (nine *v* 15; $P = .029$).

Reasons for the lack of demonstrated effectiveness in this amifostine trial are not clear, but there are four plausible explanations for this observation. First, in comparison with other published series, this study required a more rigorous surveillance program to assess for chemotherapy-induced peripheral neuropathy; therefore, our results may represent a detection bias. Second, the underlying mechanism of paclitaxel chemotherapy–induced peripheral neuropathy may be different from that of cisplatin-induced peripheral neuropathy and not amenable to amifostine neuroprotection. Third, the statistical design set stringent criteria to determine effectiveness, such that a modest effect of amifostine may have been missed, resulting in a false-negative study. Last, it may be that amifostine is not effective in preventing the development of peripheral neurotoxicity in patients receiving cytotoxic chemotherapy.

The prescribed chemotherapy regimen of cisplatin 75 mg/m^2 plus paclitaxel 175 mg/m^2 administered over 3 hours was useful for the study of amifostine neuroprotection because of its associated high incidence of peripheral neuropathy. However, carboplatin has largely replaced cisplatin as the preferred plati-

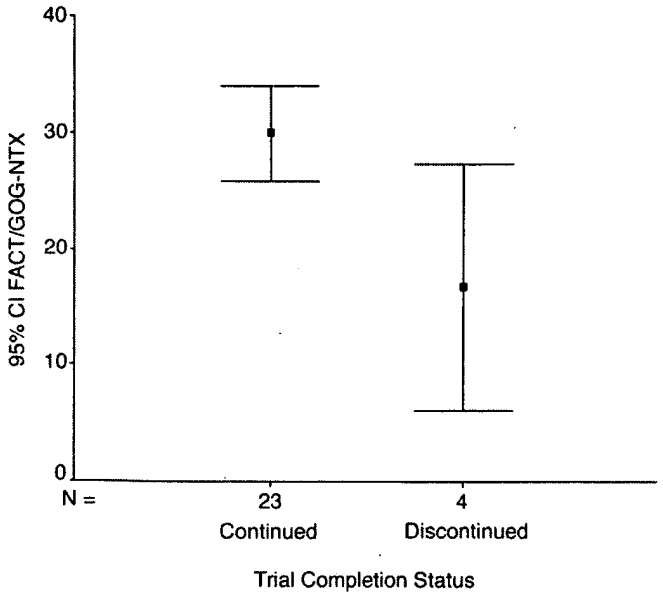

Fig 4. 95% Confidence intervals for Functional Assessment of Cancer Therapy/ Gynecologic Oncology Group-Neurotoxicity (FACT/GOG-Ntx).

num analog for treatment of ovarian cancer because of its perceived equivalent cytotoxicity and more favorable toxicity profile, including less severe nausea, emesis, nephrotoxicity, and neurotoxicity. Also, although paclitaxel is still the favored taxane in most clinical trials, docetaxel seems to be a suitable alternative, with less neurotoxicity, more myelosuppression, and no substantial difference in activity.[23]

At baseline, before each treatment cycle, and 3 months after completing treatment, a clinical evaluation for neuropathic sensory toxicity was conducted and graded using the NCI CTC scale. Patients also completed the Ntx subscale questionnaire and underwent VPT testing. Postma et al[28] studied the CTC system for grading chemotherapy-induced peripheral neuropathy. For all grades of neurotoxicity, the inter-observer agreement using the CTC scale was 46%. The inter-observer agreement for neurotoxicity grade ≤ 2 versus 3 was 81%. Comparing the CTC system with other commonly used grading systems, the authors recommended caution when interpreting results across studies, which use different scales for neurotoxicity grading.

Although VPT testing is considered to be the gold standard, it is not without inaccuracies. Ideally, patients should undergo VPT testing under identical circumstances: time of day, nondistracting environment, room location, and ambient temperature. Skewed results may occur if the same finger and toe are not used at each testing procedure, the patient comes into contact with the metal casing of the vibrating rods, or the patient is not prevented from viewing the instrument settings or data sheet during the testing procedure. Devices used for VPT testing are not inexpensive and must be calibrated every 6 months.

An important finding in this trial was the clinical validation of the FACT/GOG-Ntx subscale. Neurotoxicity evaluations by physicians are notoriously inconsistent, and a validated, patient-reported scale is an important contribution to the future study of chemotherapy-induced peripheral neuropathy. Other validated neurotoxicity questionnaires exist and have been used by clinical trials groups.[29] All four patients with dose-limiting ($\geq$ grade 2) neurotoxicity had Ntx scores less than 25. Pending further study, this score should be considered indicative of chemotherapy-induced peripheral neuropathy. On the basis of the ROC analysis, it would seem that vibration perception threshold measurements are less sensitive to the development of peripheral neuropathy. Future clinical studies of chemotherapy-induced neurotoxicity may rely on the Ntx subscale.

Amifostine's level of activity in this trial was insufficient to warrant further study in a phase III trial. Glutamine has been reported to decrease the potential for paclitaxel-induced neuropathies.[30] Future clinical strategies to reduce the incidence of chemotherapy-induced neuropathy should investigate glutamine or one of the other emerging neuroprotectors.

APPENDIX

The appendix is included in the full-text version of this article, available on-line at www.jco.org. It is not included in the PDF (via Adobe® Acrobat Reader®) version.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Acted as a consultant within the last 2 years: David H. Moore, Lilly Oncology, Schering-Plough, GlaxoSmithKline.

REFERENCES

1. Ehrlich CE, Einhorn L, Williams SD, et al: Chemotherapy for stage III-IV epithelial ovarian cancer with *cis*-dichlorodiammineplatinum (II), adriamycin, and cyclophosphamide: A preliminary report. Cancer Treat Rep 63:281-288, 1979

2. Omura GA, Blessing JA, Ehrlich CE, et al: A randomized trial of cyclophosphamide and doxorubicin with or without cisplatin in advanced ovarian carcinoma: A Gynecologic Oncology Group study. Cancer 57:1725-1730, 1986

3. Neijt JP, ten Bokkel Huinink WW, van der Burg ME, et al: Randomised trial comparing two combination chemotherapy regimens (Hexa-CAF vs CHAP-5) in advanced ovarian carcinoma. Lancet 2:594-600, 1984

4. Advanced Ovarian Cancer Trialists Group: Chemotherapy in advanced ovarian cancer: An overview of randomized clinical trials. BMJ 303:884-893, 1991

5. Donehower RC, Rowinsky EK, Grochow LB, et al: Phase I trial of taxol in patients with advanced cancer. Cancer Treat Rep 71:1171-1177, 1987

6. Trimble EL, Adams JD, Vena D, et al: Paclitaxel for platinum-refractory ovarian cancer: Results from the first 1,000 patients registered to National Cancer Institute Treatment Referral Center 9103. J Clin Oncol 11:2405-2410, 1993

7. Rowinsky EK, Gilbert MR, McGuire WP, et al: Sequences of taxol and cisplatin: A phase I and pharmacologic study. J Clin Oncol 9:1692-1703, 1991

8. McGuire WP, Hoskins WJ, Brady MF, et al: Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and IV ovarian cancer. N Engl J Med 334:1-6, 1996

9. Lo Monaco M, Milone M, Batocchi AP, et al: Cisplatin neuropathy: Clinical course and neurophysiological findings. J Neurol 239:199-204, 1992

10. Rowinsky EK, Donehower RC: Paclitaxel (Taxol). N Engl J Med 332:1004-1014, 1995

11. Sarosy G, Kohn E, Stone DA, et al: Phase I study of taxol and granulocyte colony-stimulating factor in patients with refractory ovarian cancer. J Clin Oncol 10:1165-1170, 1992

12. Markman M, Connelly B, Kennedy A, et al: Cisplatin (75 mg/m^2) plus 3-hour infusional taxol (135 or 175 mg/m^2): Unexpected high incidence of peripheral neuropathy. Gynecol Oncol 60:98-99, 1996 (abstr 8)

13. Eisenhauer EA, ten Bokkel Huinink WW, Swenerton KD, et al: European-Canadian randomized trial of paclitaxel in relapsed ovarian cancer: High-dose versus low-dose and long versus short infusion. J Clin Oncol 12:2654-2666, 1994

14. Piccart MJ, Bertelsen K, James K, et al: Randomized intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer: Three-year results. J Natl Cancer Inst 92:699-708, 2000

15. Yuhas JM, Culo F: Selective inhibition of the nephrotoxicity of *cis*-DDP by WR-2721 without altering its antitumor properties. Cancer Treat Rep 64:57-64, 1980

16. Muller LJ, Moorer-van Delft CM, Roubos WE, et al: Quantitative ultrastructural effects of cisplatin (Platinol), carboplatin (JM8), and iproplatin (JM9) on neurons of freshwater snail *Lymnaea stagnalis*. Cancer Res 52:963-973, 1992

17. Mollman JE, Glover DJ, Hogan WM, et al: Cisplatin neuropathy: Risk factors, prognosis, and protection by WR-2721. Cancer 61:2192-2195, 1988

18. Rose P, Kemp G, Glick J: Ethyol (amifostine) protects against cumulative cisplatin toxicities. Proc Am Soc Clin Oncol 15:533, 1996 (abstr 1724)

19. Cella D: F. A. C. I. T. Manual: Manual of the Functional Assessment of Chronic Illness Therapy (FACIT) Scales (version 4). Evanston, IL, Center on Outcomes, Research and Education (CORE), Evanston Northwestern Healthcare and Northwestern University, 1997

20. Cella D, Peterman A, Hudgens S, et al: Measuring the side effects of taxane therapy in oncology: The Functional Assessment of Cancer Therapy-Taxane (FACT-Taxane). Cancer 98:822-831, 2003

21. Calhoun EA, Welshman EE, Chang C-H, et al: Psychometric evaluation of the Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity (FACT/GOG-Ntx) questionnaire for patients receiving systemic chemotherapy. Int J Gynecol Cancer (in press)

22. Hay JW: Quality of life effects of chemotherapy-induced neuropathy in ovarian cancer. Proc Am Soc Clin Oncol 21:222, 2002 (abstr 886)

23. Vasey PA: Survival and longer-term toxicity results of the SCOTROC study: Docetaxel-carboplatin vs. paclitaxel-carboplatin in epithelial ovarian cancer—Scottish Gynaecological Cancer Trials Group. Proc Am Soc Clin Oncol 21:202a, 2002 (abstr 804)

24. Glover D, Glick J, Weiler C, et al: Phase I trials of WR-2721 and cis-platinum. Int J Radiat Oncol Biol Phys 10:1781-1784, 1984

25. Glover D, Glick JH, Weiler C, et al: Phase I/II trials of WR-2721 and cis-platinum. Int J Radiat Oncol Biol Phys 12:1509-1512, 1986

26. Glover D, Glick J, Weiler C, et al: WR-2721 and high-dose cisplatin: An active combination in the treatment of metastatic melanoma. J Clin Oncol 5:574-578, 1987

27. Kemp G, Rose P, Lurain J, et al: Amifostine pretreatment for protection against cyclophosphamide-induced and cisplatin-induced toxicities: Results of a randomized control trial in patients with advanced ovarian cancer. J Clin Oncol 14:2101-2112, 1996

28. Postma TH, Heimans JJ, Muller MJ, et al: Pitfalls in grading severity of chemotherapy-induced peripheral neuropathy. Ann Oncol 9:739-744, 1998

29. Cassidy J, Paul J, Soukop M, et al: Clinical trials of nimodipine as a potential neuroprotector in ovarian cancer patients treated with cisplatin. Cancer Chemother Pharmacol 41:161-166, 1998

30. Peltier AC, Russell JW: Recent advances in drug-induced neuropathies. Curr Opin Neurol 15:633-638, 2002